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(54) **Liposomal compositions and processes for their production.**

(57) Liposomal compositions are described containing an enzyme having L-Asparaginase activity characterized by having a protein/lipid ratio of at least 30 μg/ μmol, the size of liposomes being up to 1000 nm. The enzymatic activity is located in the aqueous or lipid phase or both. The compositions are prepared by forming multilamellar liposomes containing the enzyme and subjecting the liposomes to lyophilization, rehydration and extrusion under pressure.

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This invention relates to liposomal compositions containing enzymes having asparaginase activity.

L-Asparaginase (L-asparaginase; L-asparagine amido hidrolase E.C.3.5.1.1.) is an effective anti tumor agent active against human acute lymphoblastic leukemia (1). These malignant cells have a metabolic defect in L-asparagine synthetase being dependent on an exogenous supply of L-asparagine. The mode of action of L-asparaginase is based on starvation to death of malignant cells by degradation of their specific substrate. The intravenous administration of free enzyme causes several side effects in humans, mainly acute allergic reactions ranging from fever, skin rashes to death secondary to anaphylactic shock, thrombosis or hemorrhage (2,3).

To partially overcome these limitations, several strategies can be followed such as the inclusion of proteins into liposomes, with the aim of rendering the protein non immunogenic and increasing its biological half-life in the organism.

In the past few years, liposomes have been extensively studied as carriers of traditional drugs (4, 5, 6, 7) and to a smaller extent of relatively new drugs, namely peptides and proteins (8). The increasing availability of pure recombinant proteins, their approval as drugs and the development of liposome technology capable of preserving protein structure (8, 9), seems to open new perspectives to the use of liposomal formulations of proteins as therapeutic agents. This approach has already been used with INF (10), INF (11), IL-1 (12), Glucose-6-Phosphate dehydrogenase (13), Factor VIII (9) and antigens (14).

The potential advantage of the use of liposomes as carrier system of L-asparaginase for medical applications, is the reduction of allergic reactions, as antigen determinants of the enzyme are masked by encapsulation (15).

Liposomes have been proposed as carriers of L-asparaginase and have shown evidence of protection against the allergic reactions. Analysis of the current literature indicates that although the encapsulation of L-asparaginase in liposomes has attracted attention in the past (15, 16) and in recent years (17, 18), biologically effective systems have not been achieved either from the galenic or the pharmacological point of view.

The present invention is addressed to the problem of preparing liposomes with efficient encapsulation of L-Asparaginase in native form, hydrophobized form or both. These liposomal preparations are required to have the following characteristics: preservation of active enzyme, high entrapment efficiency, prolonged serum half life, reduced toxicity compared with the free enzyme and high antitumoral activity.

It has been found that liposomal compositions meeting the above requirements are those which have the parameters and are prepared by the methods described hereinafter.

The present invention comprises a liposomal composition containing an enzyme having L-Asparaginase activity characterised by having a protein/lipid ratio of at least 30μg/μmol, the size of liposomes being up to 1000 nm. Preferably the size of the liposomes is no greater than 800 nm, a most advantageous size range being from 100 to 200 nm. It will be understood that compositions according to the invention consist at least predominantly of liposomes having the specified maximum size. The required liposome size is achieved by means of filters of the appropriate pore size as is well understood in the art.

Liposome compositions in accordance with the present invention utilse native Asparaginase, which is hydrophilic and therefore dissolved in the aqueous phase, or a hydrophobic derivative of the native enzyme which is therefore located in the lipid phase e.g. an acylated derivative having an acyl group containing from 8 to 18 carbon atoms such as palmitoyl. If desired, both forms of the enzyme may be used distributed in both the aqueous and oil phases of the liposome.

In order to ensure the desired proportions of protein and lipid in the final formulations it is desirable, when preparing the liposomes, to start with an initial protein/lipid ratio of at least 40 μg/μmol (0.06 mg protein/1mg lipid) or 80 μg/μmol (0.11 mg rotein/1 mg lipid), respectively for hydrophobised or native enzyme.

Liposome compositions may be prepared, according to this invention, by a general process which will be explained hereinafter in flow sheet diagrammatic form. The general process is adaptable depending on whether native or hydrophobised enzyme is used. In general the process comprises forming multilamellar liposomes containing the enzyme and subjecting the liposomes to lyophilisation, rehydration and extrusion under pressure. For native enzyme the process is characterised by forming multilamellar liposomes from an aqueous solution of the enzyme and the lipid, lyophilising the resulting liposomes, rehydrating the lyophilised liposomes by treatment with a small volume of non-saline suspending fluid followed, after a resting phase, by treatment with a saline solution, diluting the resulting suspension and extruding the suspension under pressure without prior separation of non-incorporated enzyme.

For hydrophobised enzyme the multilamellar liposomes are formed as empty liposomes and a hydrophobic L-Asparaginase derivative is added as dry solid to the liposomes before the lyophilisation stage.

Liposome compositions and processes for their preparation by this invention are characterised by the use of any of the following lipids, hydrogenated or not, individually or in mixtures, in a molar ratio from 0.01 to 10: distearoylphosphatidylcholine (DSPC), phosphatidylcholine (PC), cholesterol (Chol) or its derivatives, sphyn-

gomyelin (SM), dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylglycerol (DOPG), phosphatidylglycerol (PG), dimirystoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), gangliosides ceramides, phosphatidylinositol (PI), phosphatidic acid (PA), dicetylphosphate (DcP), dimirystoylphosphatidylglycerol (DMPG), stearylamine (SA), dipalmitoylphosphatidylglycerol (DPPG) and other synthetic lipids.

The improved liposomal formulations of L-asparaginase activity makes use of extruded vesicles (VET) for both forms of the enzyme. Although showing smaller encapsulation parameters than multilamellar vesicles (DRV), the liposomes prepared by the present process are highly effective to degrade circulating substrates as, similarly to other kind of small vesicles (19), VET liposomes have been found to circulate for more prolonged periods of time than the multilamellar vesicles. No data are available in the prior literature for the encapsulation of native L-asparaginase in VET.

According to the present invention liposomes are prepared by the dehydration and rehydration of multilamellar vesicles (MLV) containing intra and extra liposomal L-Asparaginase in a solution of 0.3 OSM. The so formed liposomes are then resuspended in a volume 20 times higher than the final volume and submitted to several extrusions through polycarbonate filters of pore size from 5.0 to 0.1 μm or lesser. To wash the extraliposom material and to concentrate liposomes, an ultracentrifugation is carried out in solutions of desired osmolarity, generally 0.3 OSM, obtaining the final liposomal preparation in the desired volume.

The so prepared extruded vesicles show a high retention of specific enzymatic activity (73 to 99%) and Encapsulation Efficiency (E.E.) up to 28%, according to lipid composition and size. The P/L ratio in liposomes is at least 32 μg/ μmol of lipid, being also size and composition dependent (Table I).

The higher stability in human serum observed for VET containing L-Asparaginase when compared with DRV elucidates the importance of size. In fact, the stability in human plasma of liposomes type DRV was increased from 65 to 95% after size reduction by extrusion. Although it seems important to avoid the existence of osmotic gradients after liposome in vivo administration, little attention has been given up to now, to the effects of solute osmolarity on protein encapsulation. The method usually described in literature of using 0.154 M NaCl to keep osmolarity, resulted in a decrease in the E.E. with all lipid compositions tested. By rehydration of liposomes with mannitol, as done by the present method, there was no effect on all the parameters under study. The liposomes are easily centrifuged and physiological osmolarity can be kept after reconstitution.

Liposomal formulations of L-Asparaginase herein described are significantly less toxic than free L-Asparaginase, as concluded by the increase of animals survivals from 83 to 100%, when both forms of enzyme are administered (Table II). The present formulations of L-Asparaginase show enhanced antitumoral activity as compared to free enzyme or to multilamellar liposomes (DRV), also tested (Table IV). These results are according to the pharmacokinetic data shown in Table III: liposomes prepared by the present invention increases 4.5 to 14.5 fold the half life of free enzyme, depending on the lipid composition used. The fact that the encapsulated enzyme circulates longer than the free one increases the pharmacological effects continually degrading the circulating substrate L-asparagine.

Alternatively to the use of liposomal native L-Asparaginase described above, an hydrophobic derivative of the enzyme may be incorporated in the lipid matrix of the liposomes in amounts suitable for therapeutical purposes.

According to the present invention, the incorporation of the modified protein is carried out by the addition of dried hydrophobized L-asparaginase to multilamellar liposomes (MLV) obtained by drying under nitrogen stream the necessary amount of lipid followed by hydration of the lipidic film to form MLV. Dehydration and rehydration in solutions with osmolarity of 0.3 OSM is the next step. The so formed liposomes are then resuspended in a volume 20 times higher than the final volume and submitted to several extrusions through polycarbonate filters of pore size from 5.0 to 0.1 um or lesser. An ultracentrifugation in gradients, with a composition being dependent of the lipidic composition of the liposomes, allows to collect liposomes in the middle of the gradient and neglecting the non-incorporated protein that accumulates at the bottom of the gradient. To wash the gradient substance out of the liposomal media, a last ultra-centrifugation is carried out in solutions of desired osmolarity, generally 0.3 OSM, obtaining the final liposomal preparation in the desired volume.

In prior methods described in the literature, the addition of modified protein is done by means of a solution to a film of dried lipids forming liposomes with encapsulated protein (20). Some authors do a lyophilization of these solutions followed by a hydration (21). These processes are substantially different from that of the present invention because, in the present process, the protein addition is carried out in a lyophilized form into preformed liposomes. Also, no other author uses the ultracentrifugation technique in gradients to separate the non-incorporated protein. There are no data in the literature concerning extrusion techniques applied to hydrophobic proteins incorporated in liposomes. The results of the present process are much improved in terms of recovery, encapsulation efficiency, protein concentration and biological activity. By this process, liposomes with great catalytic activity and great circulating time are obtained. Furthermore, Acyl-L-Asparaginase has not hitherto

been encapsulated in such large amount of final protein per mass of lipid organized in liposomes.

Small unilamellar liposomes prepared by sonication of multilamellar vesicles have been explored in the past as carriers of hydrophobic derivatives of L-asparaginase by superficial insertion of the acylated enzyme in the outer layer of liposomes, but without success (22). The efficiency of the process was extremely low (incorporation efficiency from 3 to 4%), leading to preparations unsuitable for intravenous administration, as high doses were needed to reach an acceptable antitumor activity.

Incorporation of hydrophobized L-Asparaginase has also been tried by us using several other prior methods namely:

1 - Incubation of small unilamellar vesicles in the presence of different concentrations of protein and different amounts and kinds of detergent. We obtained a maximal incorporation efficiency of 3% and recovery of less than 1 % of the protein initially present in the process.

2 - Use of the detergent-dialysis method for preparing large unilamellar vesicles, a method extensively used by different authors to incorporate membrane proteins in the lipid matrix of liposomes (23). With this process, and trying different amounts and kinds of detergent, and different initial protein concentrations and lipids, we obtained a maximal incorporation efficiency of 10% concomitantly with an extremely low recovery, around (0.5 to 3%), of the initial protein used in the process.

3 - Use of large unilamellar vesicles obtained by the reverse evaporation method (24), comparatively using several different protein concentrations and different conditions to remove the solvent. With this method we obtained a maximal incorporation efficiency of less than 1%. with a recovery of the initial protein used in the process smaller than 0.5%.

4 - Use of multilamellar vesicles obtained by the dehydration rehydration method as described in the literature (15). We obtained a maximal incorporation efficiency of 40% (21).

The liposomes, according to the present invention, besides their high incorporation efficiency from 50 to 98% have the capability of having 50 to 75% of the total catalytic activity in the intact form of the vesicles.

Pharmacokinetic studies clearly indicate a longer circulating time for our liposomal preparations irrespectively of the lipidic composition used, when compared to Palmitoyl-L-ASNase. Immunological response studies have provided evidence for the ability of PC:CHOL:PI liposomes to avoid the response of the immune system to the modified enzyme as shown for other systems with native asparaginase or other enzymes (15). Antitumoral studies strongly evidence the ability of the liposomes obtained by the present process to be active against tumor models in animals.

The present invention also comprises DRV and VET having in their structure simultaneously both forms of the enzyme: L-asparaginase and hydrophobized L-asparaginase. Vesicles of this kind are prepared by adding multilamellar vesicles containing native L-asparaginase to dried hydrophobized L-asparaginase. All the following steps necessary to obtain the final preparation are similar to the ones described for incorporation of hydrophobized L-asparaginase. The resulting formulations are novel and show improved and sustained release of the enzyme in vivo.

The methods, vesicle size and lipid composition here used, resulted in high E.E., high in vitro stability and no in vitro toxicity. Higher intra-liposomal concentration and stability in plasma result in higher circulation time of the enzyme and greater efficiency, as the target is a circulating substrate. The encapsulation method is extremely simple and easy to scalle up. Namely: using enzyme free MLV or MLV containing intra and extra liposomal enzyme; mannitol in the rehydration step; high volume of dilution of DRV before filtration; non washed DRV as starting material to prepare VET; abolishing the freeze-thaw steps refered in previous methods (9); using small size vesicles and lipidic mixtures of PC:Chol:SA; PC:Chol:PI and PC:Chol:GM1.

The liposomal forms of L-Asparaginase of the present invention are immunogenic safe by intravenous injection and evidence higher therapeutic activity.

METHOD

The liposomal product is prepared according to a general scheme for the incorporation either of the native form of L-Asparaginase, an hydrophobized form of L-Asparaginase, or both simultaneously, respectively in the internal aqueous internal space of liposome, or in the lipid matrix of liposomes or in both. A general scheme of the method is presented as Figure 3 followed by detailed description in text.

The method consists on the following steps:

1.The lipid mixture is dried.

2.The lipidic film is hydrated with water or an aqueous solution containing protein.

3.The formed multilamellar liposomes, alone or mixed with lyophilized protein, are dehydrated.

4.The resulting powder is rehydrated with an aqueous solution.

5.The suspension is filtered under pressure.

6.The non-encapsulated material is separated from liposomes by ultracentrifugation, in a gradient medium if necessary.

7.The final liposomes are concentrated by ultracentrifugation.

According to protein solubility, options a), b) or c) are used:

a)In case of water soluble protein:

- the addition of protein to lipid is done in step 2.
- the formed liposomes are lyophilized alone (step 3.)
- there is no need of a gradient separation.

b)In case of the hydrophobized protein:

- the lipidic film is hydrated with water.
- the addition of protein to lipid is done, in a lyophilized form, in step 3.
- the non-incorporated material is ultracentrifugated in a gradient medium.

c) In case of a mixture of native and hydrophobized protein:

- The lipidic film is hydrated with an aqueous solution containing protein.
- The formed liposomes (MLV), mixed with lyophilized protein, are dehydrated.
- The non-encapsulated material is separated from liposomes by ultracentrifugation, in a gradient medium.

Method for incorporation of native L-Asparaginase:

The method consists on the following steps:

1.The lipid mixture (1-100 mg/ml) is dried under vacuum in a N2 stream;

2.The lipidic film is hydrated with an aqueous solution of protein (0.2 - 2.0 mg/ml), slowly, with gentle stirring to form multilamellar vesicles; the protein to lipid ratio should be higher than 80 µg/µmol (0.11 mg protein / 1 mg lipid).

3.The mixture is lyophilized on a freeze-drier under vacuum (25 mtorr during, at least, 4 hours);

4.The lyophilized powder is rehydrated in two steps: first with a 1/5 - 1/10 of the initial volume of a solution of osis with vigorous vortexing followed by 30 min. rest at temperature 10 C above the phase transition temperature of the lipid, secondly the volume is brought up to the initial volume with 0.154 M NaCl;

5.The resulting mixture is then 10 fold diluted with 0.154M NaCl and extruded under pressure through polycarbonate filters of controlled pore size, successively 1000nm, 800nm, 200nm and 100nm, 2 times each, using an Extruder device;

6.The extruded mixture is ultracentifuged at 270,000 g during 90min. The pellet, consisting of liposomes containing encapsulated L-Asparaginase is resuspended in an appropriate volume of 0.154 M NaCl.

Method for incorporation of hydrophobized form of L-Asparaginase:

The method consists of the following steps:

1.The lipid mixture (1 - 100 mg/ml) is dried under vacuum in a N2 stream;

2.The lipidic film is hydrated with 1ml of water, slowly, with gentle stirring to form multilamellar vesicles.

3.The multilamellar vesicles are added to protein in lyophilized form (0,2 - 100 mg); the protein to lipid ratio should be higher than 40 µg/ (0.06 mg protein / 1 mg lipid). This suspension is gently mixed until the powder of protein is homogeneously dispersed into the suspension of liposomes.

4.The mixture is lyophilized on a freeze-drier under vacuum (25 mtorr during, at least, 4 hours).

5.Rehydration of the lyophilized powder is performed in two steps: firstly with 100 ul to 250 ul of a solution of osis with vigorous vortexing followed by 30 min rest at a temperature 10 C above the phase transition temperature of the lipid, secondly the volume is brought up to the initial 1 ml volume with 0.154 M NaCl.

6.The resulting mixture is then 10 fold diluted with 0. 154M NaCl and extruded under pressure through polycarbonate filters of control led pore size, successively 5.0 µm, 2.0 µm, 1.0 µm, 0.8 µm, 0. 6 µm, 0. 4 µm and 0. 2 µm, stopping on the filter pore size necessary to produce liposomes of the required diameter, using an Extruder device.

7.The extruded mixture is ultracentifuged at 45,000 rpm during 45 min in a sacarose gradient, collecting the liposomes at the middle of the gradient and neglecting the protein in the bottom of the gradient. To wash the sacarose out of the liposomes and to concentrate the liposome preparation a final ultracentrifugation, at 270.000 g during 60 min, is done. The pellet, consisting of liposomes containing incorporated hydrophobized L-Asparaginase is resuspended in 1 ml of 0.154 M NaCl

Method for incorporation of both native and hydrophobized form of L-Asparaginase:

The methods for incorporation of both forms of L-Asparaginase is identical to the method of incorporation of the hydrophobized L-Asparaginase with a single difference: in step 2, instead of hydrating the lipidic film with water, the hydration is carried out with an aqueous solution of L-Asparaginase (0.2 - 2 mg/ml).

CHEMICAL AND BIOLOGICAL CHARACTERIZATION

Here are presented chemical and biological characterizations of this invention, for extruded liposomes (type VET) made from different lipid compositions, as compared with those liposome obtained by another technique (DRV Dehydration-Rehydration Vesicles).

The yield of the process means the percentage of liposomal to initial protein concentration. Protein was estimated by Lowry method (25), after previous disruption of liposomes with Triton X and SDS.

Encapsulation efficiency (E.E.) means the ratio of the liposomal and the initial protein/lipid, in percentage. The lipidic concentration was estimated according to Fiske and Subbarow (26). The enzymatic activity was estimated according to Jayaram et al (27).

A -NATIVE FORM OF L-ASPARAGINASE

1.-Encapsulation of native L-Asparaginase in liposomes

Table I shows encapsulation parameters of Liposomes type VET as compared to liposomes type DRV for initial protein concentration of 2 mg/ml and three different lipid compositions.

Table I

EFFECT OF LIPOSOMES SIZE ON ENCAPSULATION EFFICIENCY

Preparation Number	Type Liposomes	Lipid Composition	Diameter (nm)	Pi/Li (ug/umol)	Pf/Lf (ug/umol)	E.E. (%)	Recovery (%)	Activity (%)
1	DRV	PC:CHOL:SA	1250±250	131±6	96±10	73±7	61±7	99±1
2	DRV	PC:CHOL:PI	699±245	96±4	66±11	69±14	45±3	99±1
3	DRV	PC:CHOL:GM1	501±126	126±4	116	72	40	86
4	VET 200	PC:CHOL:SA	250±150	86±11	44±4	52±2	31±1	98±2
5	VET 100	PC:CHOL:SA	85±39	116±18	44±9	38±6	26±4	96±4
6	VET 200	PC:CHOL:PI	160±17	110±11	33±4	31±1	21±6	92±1
7	VET 100	PC:CHOL:PI	140±23	116±13	32±9	28±8	19±4	89±9
8	VET 200	PC:CHOL:GM1	-	97±4	44±2	55±14	33±10	73±4
9	VET 100	PC:CHOL:GM1	137±10	126±24	34±14	26±8	18±6	84±5

Pi/Li - means the ratio of the initial protein (Pi) to initial lipid (Li).

Pf/Lf - means the ratio of the final protein (Pf) to final lipid (Lf).

The molar ratios of lipidic constituents are the following : PC:Chol:SA (7:2:0.25); PC:Chol:PI (10:5:1); PC:Chol:GM1 (10:5:1)

Initial Protein Concentration 2 mg/ml.

The encapsulation efficiency, enzymatic activity, protein to lipid ratio and recovery are highly dependent on the type of vesicles and on the lipid composition used. Liposomes type DRV allow high encapsulation parameters of L-Asparaginase, with final protein to lipid (Pf/Lf) ratios ranging from 69 to 116 ug/ umol; EE from 69 to 73% and specific activity from 86 to 99%. The obtention of liposomes type VET resulted in the reduction of those parameters with E.E. of 28-55%; Pf/Lf ratios of 32 to 44ug/ umol. This reduction is proportional to the reduction of filter pore size.

Specific enzymatic activity was not affected by filtration. For similar initial enzyme concentration, the E.E. for extruded vesicles of PC:Chol:SA was 52% for 200 nm (prep.4) and 38% for 100 nm size (prep.5), as compared to 73% without extrusion step (prep.1). The reduction on these parameters is closely related to the reduction on the vesicle diameters after extrusion, which is of the order of 4 to 6, for 100 nm pore size (prep.1 and 5; 2 and 7; 3 and 9). The reduction of liposomes diameters will increase in vitro in vivo stability and antitumour activity.

2 -Stability of liposomes containing L-ASNase in human serum

The stability of liposomes preparations (VET and DRV) was tested in the presence of human serum (Figure 1).The stability of DRV in human serum was 65 and 55 % at 48 hours, according to lipid composition. The stability of VET preparations in human serum is higher than in DRV. Formulations composed of PC:Chol:SA and PC:Chol:PI were highly stable, with 80 to 90% of the initial activity retained after 48 hours of incubation.

3. -In vivo toxicity

Toxicity studies of formulations obtained by the present process, revealed not to generate immunological response in sensitized mice. Groups of Charles River mice were immunized with three 1M injections of free or liposomal enzymes on day 0, 10 and 20. On day 30, animals were challenged with an IV injection of 2000 U/Kg of free or liposomal enzymes.

Table II shows the toxicity of L-Asparaginase liposomal formulations. The encapsulation of L-Asparaginase by the present process efficiently reduces the toxicity, as evidenced by the increases of survivals from 83% when free enzyme is given to 100% when liposomal enzyme is given.

Table II

IN VIVO TOXICITY OF L-ASPARAGINASE PREPARATIONS

Experiment	Animals	Dead	Shock (%)
IM-Free L-Asparaginase IV-Free L-Asparaginase	6	6 (100%)	1 (17%)
IM-Free L-Asparaginase IV-Liposomal L-Asparaginase	6	0 (0%)	0 (0%)
IM-Liposomal L-Asparaginase IV-Free L-Asparaginase	6	6 (100%)	0 (0%)
IM-Liposomal L-Asparaginase IV-Liposomal L-Asparaginase	6	0 (0%)	0 (0%)

4. - Pharmacokinetics

Pharmacokinetics studies were done in Charles River mice using the liposomes type VET 100 as compared to liposomes type DRV, as an example, when the same dose was administred.

Blood was collected from rectroorbital vein at fixed times.

Activity of L-Asparaginase were determinated in total blood.

For VET liposomes the half circulation time (t 1/2) was increased from 1.9 hours (for free enzyme) to 9.3-28.7 hours. For liposomes type DRV the t 1/2 is only 0.1 hour (table III).

Table III

PHARMACOKINETICS STUDIES

Pharmacokinetic parameters obtained by mathematical treatment. Mean residence time (MRT), volume of distribution at the steady state (Vol. D. (SS)) and half life (T 1/2) are shown for each experiment. Dose equal to 800 U/Kg of weight.

Experiment	MRT (H)	Vol. D. (SS) (ml)	T 1/2 (H)
Free L-Asparaginase (Commercial)	2.9	1.3	1.9
Liposomes type DRV	0.3	3.5	0.1
Liposomes type VET (PC:Chol:SA)	11.1	1.9	9.3
Liposomes type VET (PC:Chol:PI)	15.4	1.7	11.0
Liposomes type VET (PC:Chol:GM1)	34.4	4.1	28.7

5.- Antitumour Activity

As a crucial test to evaluate the therapeutic importance of the product, in vivo studies were perfomed using BDF1 mice, bearing tumoral cells P1534. These animals were treated with the same dose, either with liposomal type VET, free commercial enzyme or another liposomal formulation (DRV).

As shown in table IV, the animals treated with VET liposomal enzyme presented higher percentage of cure. They showed survival ratios of treated / control (%) higher than 700% as compared to 135% and 392% , respectively for free and DRV liposomal enzyme.

Table IV

ANTITUMOR ACTIVITY OF LIPOSOMAL FORMULATIONS INCORPORATING L-ASPARAGINASE and FREE L-ASPARAGINASE

Dose equal to 800 U/Kg of weight

Experiment	Animals Cured %T/C
Free L-Asparaginase (Commercial)	2/10 392
Liposomes type DRV	0/10 135
Liposome type VET 100 PC:Chol:Sa	3/10 721
Liposome type VET 100 PC:Chol:PI	2/10 >700
Liposome type VET 100 PC:Chol:GM1	6/10 >764

Dose equal to 800 U/Kg of weight.

B. HYDROPHOBIZED FORM OF L-ASPARAGINASE

1. - Encapsulation of hydrophobized L-Asparaginase in liposomes

The first step to incorporate palmitoyl-L-ASNase was to determine the liposomal composition and the size of such vesicles. We found that the best formulations were PC:Chol:PI in a molar ratio of 10:5:1 and PC:Chol:SA

in a molar ratio of 7:2:0.25. Incorporation parameters were determined for these type of vesicles submitted to the extrusion procedure (VET 200) and non-submitted to the extrusion step (DRV). The recovery ranged from 36+-8 to 43+-15% to the VET 200 and from 56+-9 to 65+-7 % to the DRV. The encapsulation efficiency ranged from 69+-19 to 74+-11% (VET 200) and from 80+-16 to 86+-14 % (DRV). The ratio of intact activity to total (destroyed) activity is the most significant value as it indicates the activity of palmitoyl-L-ASNase exposed to the outer medium. It increases from about 30% in the DRV to 51 and 7 4 % in the VET 200 formulations.

In order to obtain enough amount of liposomal palmitoyl-L-ASNase to the animal studies it became necessary to increase the quantity of lipid and protein in the liposomal preparations. For that, the initial ratio of 16.5 umol/0.25 mg (lipid/protein) was modified to 66.0 umo1/2.87 mg. For the VET 200 liposomes made with PC:Chol:PI, the new incorporation parameters were 29 +-7 % for the recovery, 46+-6 % for the encapsulation efficiency, and 54+-14 % for the Intact Activity/Total activity. For the VET 200 liposomes made with PC:Chol:SA, the new incorporation parameters were 44+-6 % for the recovery, 66+-10 % for the encapsulation efficiency, and 46+-12 % for the Intact Activity/Total activity.

2. -Pharmacokinetics

The pharmacokinetics parameters of the VET 200 and of palmytoil-L-ASNase suspended in Tween 80 0.5% filtered through 0.2 um were obtained from Figure 2 and are shown in Table V. The incorporation of palmitoyl-L-ASnase in liposomes increased the mean residance time for 4.1 H to values greater than 32 H.

The Area Under Curve has also inceased significantly, from 77.4 U.H/ml to values greater than 650 U.H/ml. The plasma clearence has been reduced with the incorporation of liposomes, from 0.646 ml/H to values smaller than 0.076 ml/H. No alteration has been observed in the volume of distribution (steady-state) with the incorporation.

These pharmacokinetic studies clearly indicate a longer circulating time for the liposomal preparations independently of the lipidic composition used, when compared to Palmitoyl-L-ASNase.

3. - In vivo toxicity

The immunological studies (results presented in Table VI) show that palmitoyl-L-ASNase retains part of the toxicity of the native L-ASNase, in either the suspensions used, with 4 of the 6 animals showing anaphylatic shock and in one animal causing dead.

The liposomal formulation of PC:Chol:PI improved these results. When it is used as the sensitizating and the challenge agent, no anaphylatic shock or dead was observed. Also in the case of palmitoyl-L-ASNase as the challenge agent, animals sensitized with PC:Chol:PI liposomes show no anaphylatic shock or dead.

By another way, liposomal formulation of PC:Chol:SA have increased immunological response in either the cases, where these liposomes are used as both the sensitizing and challenge agent (all the 6 animals showing evidence of anaphylatic shock with 2 of then going to dead), and where these liposomes are used only as the sensitizing agent and Palmitoyl-L-ASNase in Tween 80 0.5% used as the challenging agent (all the 6 animals showing evidence of anaphylatic shock and 3 of them going to dead).

The immunological response studies evidenced for the ability of PC:CHOL:PI liposomes to avoid the response of the immune system to the modified enzyme as shown for other systems with native asparaginase or other enzymes (15). PC:CHOL:SA liposomes increase the immune response when compared to the non-liposomal enzyme, despite of the antitumoral activity that these type of vesicles maintain.

4. - Antitumour Activity

Table VII compares the antitumor activity of Palmitoyl-L-ASNase in Tween 80 and of the liposomal formulations, estimated as their ability to supress the growth of solid lymphoma P1534. The activity of all preparations is dose dependent. 400 U/kg is a non effective dose. The liposomal Palmitoyl-L-ASNase formulations is as effective as the free Palmitoyl-L-ASNase in the 2000 U/kg dose, both exhibiting antitumor activity, with a T/C > 700%. All the dead animals suffered of liver, pancreas and spleen metastasis with no native tumor. Histopathological studies of the liver, spleen and pancreas of the dead animals revealed the massive presence of tumor cells with almost total absence of normal cells in these organs. This is an indication that, even in the low doses, all formulations were able to supress growth of native tumor.

TABLE V

PHARMACOKINETIC STUDIES

Pharmacokinetic parameters obtained by mathematical treatment of Figure 2. Mean residence time (MRT), area under curve (AUC total), plasmatic clearence (Clp), volume of distribution at the steady state (Vol. D.(SS)) and half life (T 1/2) are shown for each experiment. Dose equal to 2000 U/Kg of weight.

	MRT (H)	AUC (U.H/ml)	Clp (ml/H)	Vol.D.(SS) (ml)	T 1/2 (H)
Palmitoyl-L-ASNase in Tween 80 0.5%	4.1	77.4	0.646	2.64	2.88
Pc:Chol:SA VET 200	32.1	656.3	0.076	2.44	24.3
Pc:Chol:PI VET 200	33.1	740.9	0.067	2.24	23.7

TABLE VI

THE EFFECT OF LIPOSOMAL COMPOSITION ON IMMUNOLOGICAL RESPONSE

The effect of liposomal formulation in the immunological response. Anaphylatic shock was noticed by means of behavior alterations

Experiment	Animals	Shock	Dead
IM-Acyl-L-ASNase, Tween 80 0.5% IV-Acyl-L-ASNase, Tween 80 0.5%	6	4 (66%)	1 (17%)
IM-Acyl-L-ASNase, 0.154 M NaCl IV-Acyl-L-ASNase, 0.154 M NaCl	6	4 (66%)	1 (17%)
IM-Acyl-L-ASNase, PC:Chol:PI(0.2 um) IV-Acyl-L-ASNase, PC:Col:PI(0.2 um)	6	0 (0%)	0 (0%)
IM-Acyl-L-ASNase, Pc:Col:PI(0.2 um) IV-Acyl-L-ASNase, Tween 80 0.5%	6	0 (0%)	0 (0%)

IM-Acyl-L-ASNase, Tween 80 0.5%			
IV-Acyl-L-ASNase, PC:Col:PI(0.2 um)	6	0	0 (0%)
IM-Acyl-L-ASNase, Tween 80 0.5%			
IV-Acyl-L-ASNase, PC:Chol:SA(0.2 um)	6	0	0 (0%)
IM-Acyl-L-ASNase, PC:Chol:SA(0.2 um)			
IV-Acyl-L-ASNase, PC:Chol:SA(0.2 um)	6	6	2 (100%) (33%)
IM-Acyl-L-ASNase, PC:Chol:SA(0.2 um)			
IV-Acyl-L-ASNase, Tween 80 0.5%	6	6	3 (100%) (50%)

TABLE VII

Antitumor activity of liposomal formulations incorporating Palmitoyl-L-Asparaginase and of Palmitoyl-L-Asparaginase solubilized in Tween 80 0.5%

Experiment	Animals cured %T/C
Palmitoyl-L-ASNase	
2000 U/Kg	9 of 10 >700
400 U/Kg	5 of 10 >700
Liposomal Palmitoyl-L-ASNase	
PC:CHOL:PI 0.8 um	
2000 U/Kg	7 of 9 >700
400 U/Kg	4 of 8 >700
PC:CHOL:SA 0.2 um	
2000 U/Kg	7 of 9 >700
400 U/Kg	4 of 8 >700
PC:CHOL:PI 0.2 um	
2000 U/Kg	7 of 9 >700
400 U/Kg	4 of 8 >700

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Claims

1. A liposomal composition containing an enzyme having L-Asparaginase activity characterized by having a protein/lipid ratio of at least 30 μg/ μmol, the size of liposomes being up to 1000 nm.

2. A composition according to claim 1 in which the size of liposomes is up to 800 nm.

3. A composition according to claim 2 in which the size of the liposomes is from 100 to 200 nm.

4. A composition according to claim 1, 2 or 3 in which the enzyme is in the aqueous phase.

5. A composition according to claim 1, 2 or 3 in which the enzyme is a hydrophobic derivative of L-Asparaginase present in the lipid phase.

6. A composition according to claim 1, 2 or 3 containing L-Asparaginase in the aqueous phase and a hydrophobic derivative of L-Asparaginase in the lipid phase.

7. A composition according to claim 5 or 6 in which the derivative of L-Asparaginase is an acylated derivative having an acyl group containing from 8 to 18 carbon atoms.

8. A composition according to claim 7 in which the derivative is Palmitoyl-L-Asparaginase.

9. A composition according to any of the preceding claims characterized by having Palmitoyl-L-Asparaginase entrapped in liposomes.

10. A process of preparing a liposomal composition according to claim 1 which comprises forming multilamellar liposomes containing the enzyme and subjecting the liposomes to lyophilization, rehydration and extrusion under pressure.

11. A process according to claim 10 in which the multilamellar liposomes are formed from an aqueous solution of the enzyme.

12. A process according to claim 10 in which the multilamellar liposomes are formed as empty liposomes and a hydrophobic L-Asparaginase derivative is added as dry solid to the liposomes before the lyophilization stage.

13. A process according to claim 12 in which the solid derivative is added in the form of lyophilized material.

14. A process according to any of claims 10 to 13 in which any enzymic material not incorporated into the liposomes is removed before or after the extrusion stage.

15. A process for preparing a liposomal composition containing L-Asparaginase characterized by forming multilamellar liposomes from an aqueous solution of the enzyme and the lipid, lyophilizing the resulting liposomes, rehydrating the lyophilized liposomes by treatment with a small volume of a non-saline suspending fluid followed, after a resting phase, by treatment with a saline solution, diluting the resulting suspension and extruding the suspension under pressure without prior separation of non-incorporated enzyme.

16. A process of incorporation of a hydrophobic protein into liposomes characterized by adding the protein in dry form to preformed multilamellar liposomes which are either empty liposomes or contain a hydrophilic protein in their aqueous phase, lyophilizing the mixture and rehydrating the lyophilized material to reconstitute liposomes, and filtering under pressure to reduce size.

17. Process according to claim 16, in which any non-incorporated enzyme is separated from the reconstituted liposomes by gradient centrifugation.

18. A process for preparing a liposomal composition containing L-Asparaginase and Palmitoy-Asparaginase according to claim 16 or 17 in which the preformed multilamellar liposomes contain L-Asparaginase.

19. Liposome compositions according to any of claims 1 to 9 when prepared by a process according to any of claims 10 to 18.

20. Liposome compositions and processes for their preparation according to any of the preceding claims characterised by the use of any of the following lipids, hydrogenated or not, individually or in mixtures, in a molar ratio from 0.01 to 10: distearoylphosphatidylcholine (DSPC), phosphatidylcholine (PC), cholesterol (Chol), or its derivatives, sphingomyelin (SM), dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylglycerol (DOPG), phosphatidylglycerol (PG), dimirystoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), gangliosides, ceramides, phosphatidylinositol (PI), phosphatidic acid (PA), dicetylphosphate (DcP), dimirystoylphosphatidylglycerol (DMPG), stearylamine (SA), dipalmitoylphosphatidylglycerol (DPPG) and other synthetic lipids.

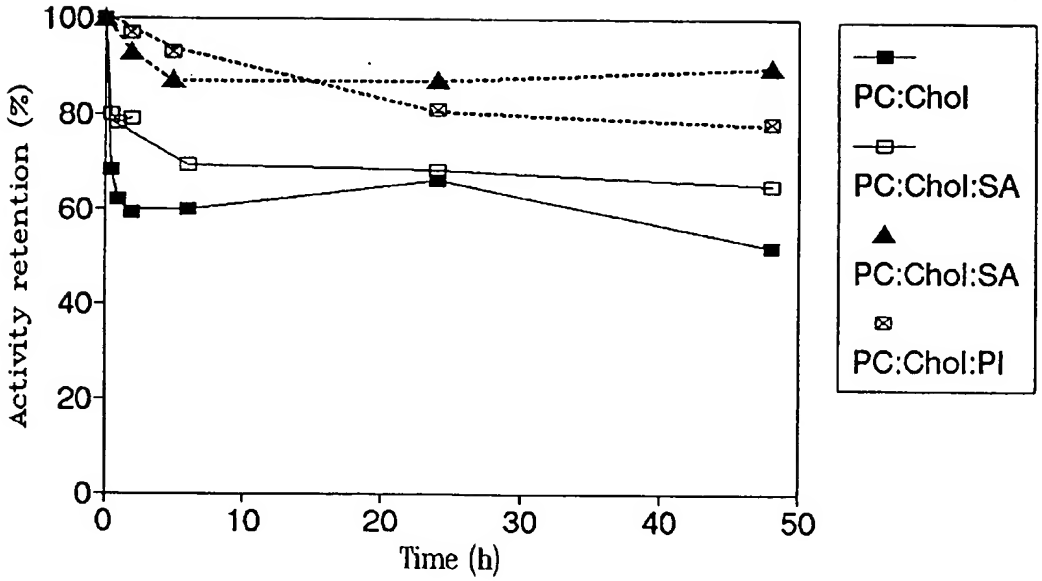


Figure 1 – Stability of liposomes DRV and VET in human serum at 37 C. The retention of activity after appropriate time periods was assayed for different lipid compositions:
DRV (Full line) – PC:Chol (1:1) ; PC:Chol:SA (7:2:0.25) ;
VET (Dotted line) – PC:Chol:SA (7:2:0.25) ; PC:Chol:PI (10:5:1)

DATA OF FIGURE 1:

STUDY OF LIPOSOMAL STABILITY IN HUMAN SERUM

	Liposomes type DRV		Liposomes type VET	
TIME (H)	PC:Chol	PC:Chol:SA	PC:Chol:SA	PC:Chol:PI
0	100	100	100	100
0.5	68	80		
1	62	78		
2	59	79	93	97
5			87	93
6	60	69		
24	66	68	87	81
48	52	65	90	78

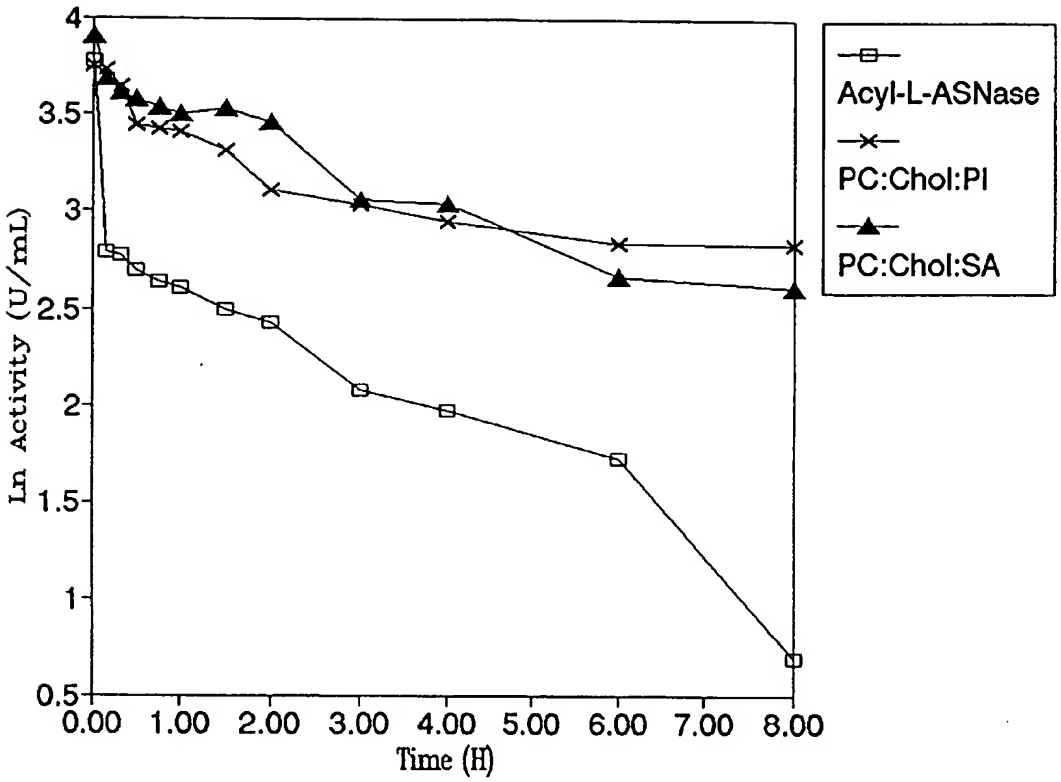


FIGURE 2 - Pharmacokinetic studies with hydrophobized formulations

DATA FOR FIGURE 2
Graphic of Ln M (media) vs. time

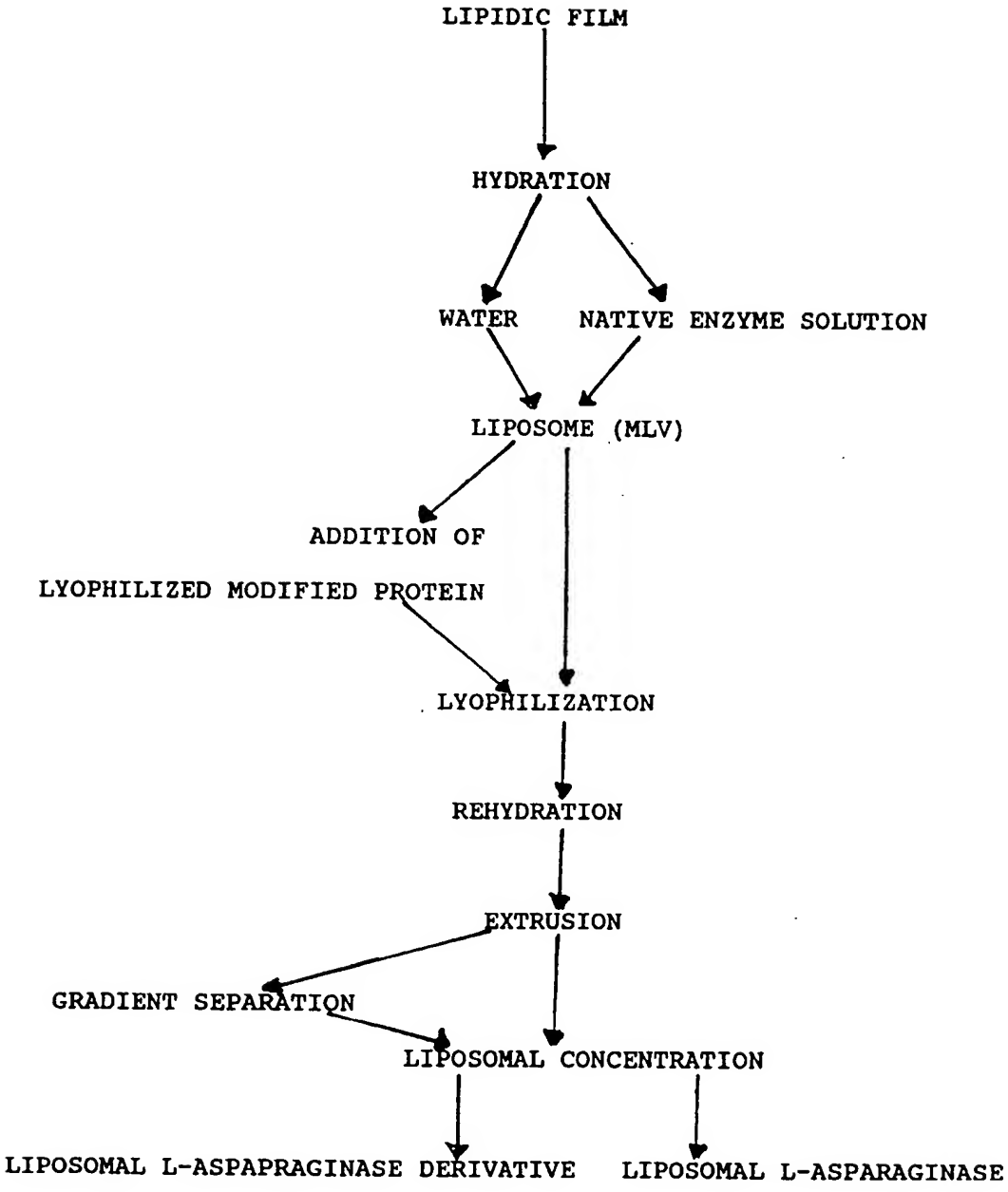
Acyl-L-ASNase

time (H)	M	M+sd	M-sd	Ln M	Ln M+sd	Ln M-sd
0.016667	43.6	48.8	38.2	3.775057	3.88773	3.642836
0.166667	16.2	18.6	14	2.785011	2.923162	2.639057
0.333333	16	17.6	14.4	2.772589	2.867899	2.667228
0.5	14.8	15.2	14.4	2.694627	2.721295	2.667228
0.75	14	16.2	11.8	2.639057	2.785011	2.4681
1	13.6	15.2	12	2.61007	2.721295	2.484907
1.5	12.2	14.8	9.4	2.501436	2.694627	2.24071
2	11.4	13.2	9.4	2.433613	2.580217	2.24071
3	8	10.4	5.4	2.079442	2.341806	1.686399
4	7.2	8.4	5.8	1.974081	2.128232	1.757858
6	5.6	7.8	3.4	1.722767	2.054124	1.223775
8	2	2.8	1	0.693147	1.029619	0

Pc:Chol:Pi

time (H)	M	M+sd	M-sd	Ln M	Ln M+sd	Ln M-sd
0.016667	42.7512	42.9272	42.5752	3.755397	3.759506	3.751272
0.166667	41.4888	52.5826	30.395	3.725424	3.962385	3.414278
0.333333	38.1814	43.2228	33.14	3.642348	3.766368	3.500741
0.5	31.3274	36.7218	25.9328	3.444493	3.603371	3.255509
0.75	30.5704	37.4184	23.7224	3.420032	3.622163	3.16642
1	30.2514	33.1826	27.3202	3.409542	3.502026	3.307626
1.5	27.5086	28.699	26.318	3.314499	3.356862	3.270253
2	22.4584	30.227	14.6898	3.111665	3.408736	2.687153
3	20.7424	22.8408	18.644	3.03218	3.128548	2.925524
4	19.0332	21.5126	16.5538	2.946185	3.068639	2.806616
6	17.0226	20.4934	13.5518	2.834542	3.020103	2.606519
8	16.9308	17.9112	15.9504	2.829134	2.885426	2.769484

Pc:Chol:SA time (H)	M	M+sd	M-sd	Ln M	Ln M+sd	Ln M-sd
0.016667	49.6	53.8	45	3.903991	3.985273	3.806662
0.166667	39.8	42.4	37.4	3.683867	3.747148	3.621671
0.333333	37	39.8	34	3.610918	3.683867	3.526361
0.5	35.8	40.8	31	3.577948	3.708682	3.433987
0.75	34.4	38.2	30.8	3.538057	3.642836	3.427515
1	33.2	38.4	27.8	3.50255	3.648057	3.325036
1.5	34.2	36	32.4	3.532226	3.583519	3.478158
2	31.8	39.4	24.2	3.459466	3.673766	3.186353
3	21.2	22.6	20	3.054001	3.11795	2.995732
4	20.8	24.2	17.4	3.034953	3.186353	2.85647
6	14.4	15.8	12.8	2.667228	2.76001	2.549445
8	13.6	15.8	11.4	2.61007	2.76001	2.433613


LIPIDIC FILM
HYDRATION
WATER
NATIVE ENZYME SOLUTION
LIPOSOME (MLV)
ADDITION OF
LYOPHILIZED MODIFIED PROTEIN
LYOPHILIZATION
REHYDRATION
EXTRUSION
GRADIENT SEPARATION
LIPOSOMAL CONCENTRATION
LIPOSOMAL L-ASPAPRAGINASE DERIVATIVE
LIPOSOMAL L-ASPARAGINASE

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Application Number

EP 91 31 0180

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
X	PATENT ABSTRACTS OF JAPAN, vol. 6, no. 165 (C-121)[1043], 28th August 1982; & JP-A-57 82 311 (TANABE SEIYAKU K.K.) 22-05-1982 * Abstract * ---	1-4	A 61 K 9/127 A 61 K 37/54
X	CHEM. PHARM. BULL., vol. 32, no. 6, June 1984, pages 2442-2445; T. OHSAWA et al.: "A novel method for preparing liposome with a high capacity to encapsulate proteinous drugs: freeze-drying method" * The whole article * ---	1-4	
Y	IDEM ---	10,11, 14,15, 19,20	
Y	JOURNAL OF MICROENCAPSULATION, vol. 7, no. 4, October/December 1990, pages 497-503; S.F. ALINO et al.: "High encapsulation efficiencies in sized liposomes produced by extrusion of dehydration-rehydration vesicles" * The whole article * ---	10,11, 14,15, 19,20	TECHNICAL FIELDS SEARCHED (Int. Cl.5) A 61 K
A	US-A-4 933 121 (LAW et al.) -----		

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	03-02-1992	BENZ K.F.

CATEGORY OF CITED DOCUMENTS

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